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PRINCIPAL INVESTIGATOR: Andrew S. Kraft, M.D.

CONTRACTING ORGANIZATION: Medical University of South Carolina
Charleston, SC 29425

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14. **ABSTRACT**
Pim is a protein kinase that has been implicated to play a role in prostate cancer. Kaplan-Meier analysis demonstrated that those patients with high Pim-1 expression are at a significantly greater risk for developing metastatic cancer. In transgenic animal models, the levels of Pim-1 protein kinase are elevated in prostate tumors caused by over expression of the c-myc ongoene, a gene over expressed in human prostate cancer. In this proposal we will examine whether Pim mimics Akt and TOR or modulates additional biochemical pathways, and use knockout mice to dissect how myc and Pim collaborate to induce transformation and growth or prostate cancers. Pim inhibitors alone or in combination with TOR inhibitors will make a powerful chemotherapeutic strategy. 15. SUBJECT TERMS Pim protein kinase, TOR, rapamycin, c-Myc, PTEN 16. SECURITY CLASSIFICATION OF: 17. LIMITATION OF ABSTRACT 18. NUMBER OF PAGES 19a. NAME OF RESPONSIBLE PERSON

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Introduction

The **purpose** of this research is to (1) determine using human prostate cancer samples whether Pim is over expressed in tumors with normal PTEN levels, (2) to decipher the biochemical mechanism of action by which Pim regulates growth of prostate cancer cells, to examine effects of Pim over expression on resistance to chemotherapy, (3) to use transgenic mice to understand whether Pim over expression is necessary for c-Myc induced growth of prostate cancer cells. The **scope** of the research involves studying normal prostate epithelia in tissue culture, malignant human prostate cancer cell lines, and nude mice with subcutaneous tumors, and transgenic mice specifically expressing these genes in mice. The research sponsored by this proposal has lead to the publication of an article in Molecular Cancer Research that details the findings discussed in the Tasks.

Task 1- The goal of this task was to determine using human prostate cancer samples whether Pim is over expressed in tumors with normal levels of PTEN. We have begun this analysis by first examining the levels of c-Myc in human prostate cancer cell lines that overexpress Pim. Our examination has taken place at two levels, the protein level and the mRNA level. First on the protein level, using western blots we find that the level of c-Myc is elevated in cell lines that overexpress Pim. This would appear to be secondary to decreased degradation. In contrast when we examined the mRNA of c-Myc in these identical cell lines the level of c-Myc was not markedly elevated. This was done by RT-PCR. Slight elevations were seen in the Pim-1 mRNA suggesting the possibility that Pim-1 and Pim-2 have different functions. These results are important because they tie c-Myc levels to the expression of Pim.

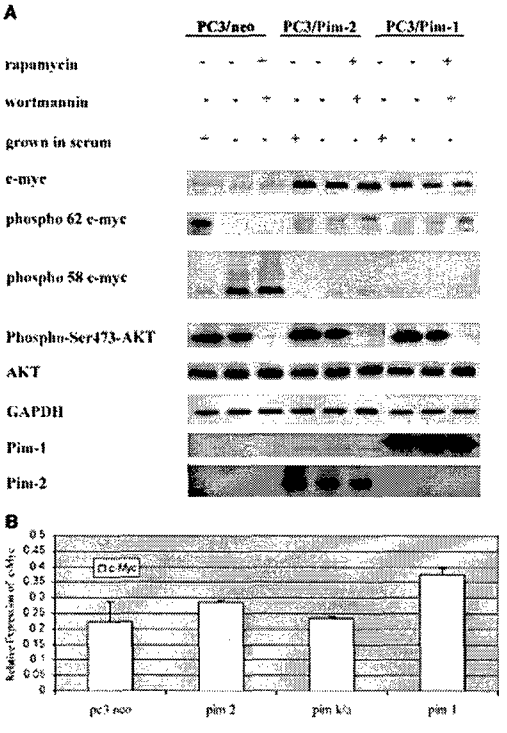


FIGURE 1. Regulation of c-Myc levels in Pim-containing PC3 cells. A. To evaluate c-Myc levels, PC3/neo, PC3/Pim-1, and PC3/Pim-2 were grown in RPMI, including 10% fetal bovine serum, then serum-starved for 24 hours and treated with rapamycin (80 nmol/L) and wortmannin (20 nmol/L) for an additional 24 hours before harvesting. Extracts were run on SDS-PAGE gels. The membrane was stripped and probed with antibodies specific for c-Myc, phospho-Ser62, phospho-Thr58, phospho-Ser473 AKT, AKT, and GAPDH, a loading control. B. To measure the level of c-Myc mRNA in PC3 cells, mRNA was extracted as described in Materials and Methods and subjected to quantitative reverse transcription-PCR. The level of c-myc mRNA was compared with GAPDH and expressed as a ratio for each sample. The experiment was repeated with three individual RNA samples and was done in triplicate. Columns, mean; bars, SD.

Task 2- The goal of the next task is to determine how Pim regulates cell proliferation, survival and growth by sharing substrates with Akt kinase. We have studied the ability of Pim to regulate the specific substrates that could play a role in the Akt pathway. We specifically looked at PP2A phosphatase. We find that prostate cancer cells that overexpress Pim have lower PP2A phosphatase activity.

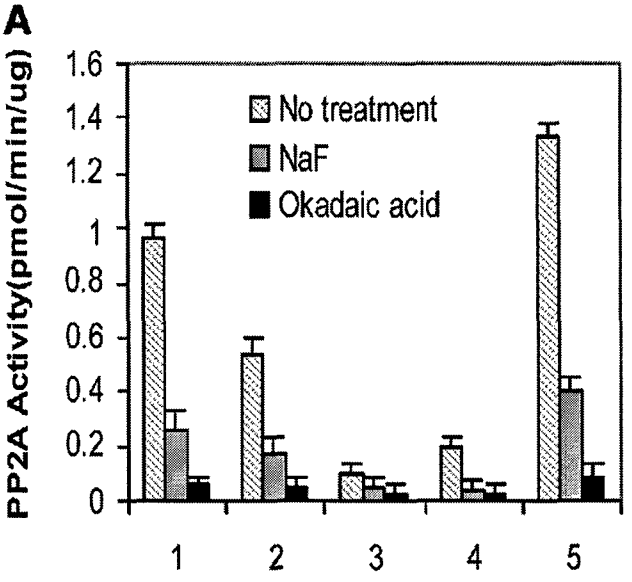


FIGURE 2A. Expression of Pim-1 or Pim-2 regulates PP2A activity in PC3 prostate tumor cells. A. Cells (2.5 x 106) were harvested in Tris-HCl–buffered saline, centrifuged, and then sonicated in 500 µL buffer [10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, and 0.02% sodium azide]. The phosphatase assay was carried out as described in Materials and Methods. As controls, either sodium fluoride (50 mmol/L) or okadaic acid (5 µmol/L) was added to cell extracts before beginning the assay. Columns, mean of triplicate determinations; bars, SD. 1, PC3/neo; 2, PC3/Pim-2 K/A; 3, PC3/Pim-2; 4, PC3/Pim-1; 5, PP2A catalytic subunit.

We find that immunoprecipitates of PP2A that the alpha and gamma subunits are dissociated. This suggests that Pim could mimic the small T antigen and cause a dissociation of PP2A into subunits and inhibit its activity

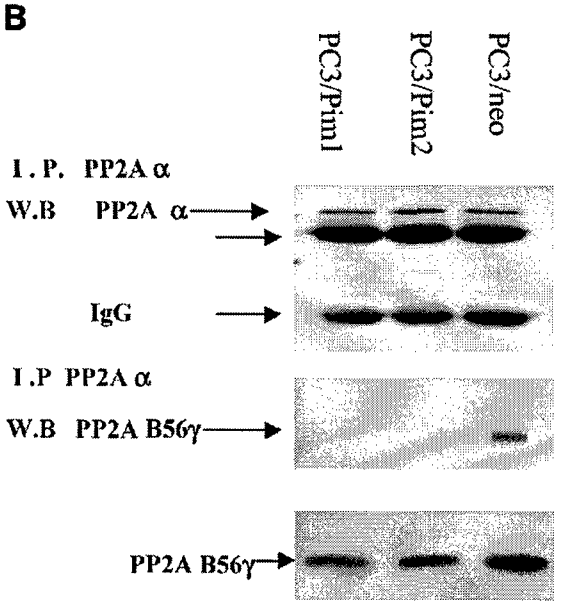


Figure 2B. 1 x 106 cells were lysed in 50 mmol/L Tris-HCl (pH 7.4); 150 mmol/L NaCl; 1 mmol/L EDTA; 1 mmol/L phenylmethylsulfonyl fluoride; and 1 µg/mL each of aprotinin, leupeptin, and pepstatin. The extracts were immunoprecipitated with an antibody to the α-subunit of PP2A. The immunoprecipitates were run on a 10% SDS-PAGE gel, transferred to polyvinylidene difluoride membranes, and probed with antibodies to the α-subunit of PP2A or an antibody to the ß-subunit B56γ. Arrows, α- and ß-subunits and the IgG light and heavy chains.

Thus Pim would effect multiple genes including c-Myc, Akt and BCR/Abl that are regulated by PP2A.

Task 3- The goal of this Task was to explore whether Pim regulates the phosphorylation patterns of 4E-BP1. In the figure below we have found that Pim regulates the phosphorylation of 4E-BP1 on p70 and p37, 46. In addition, we show that this regulation occurs when cells are starved out or treated with rapamycin.

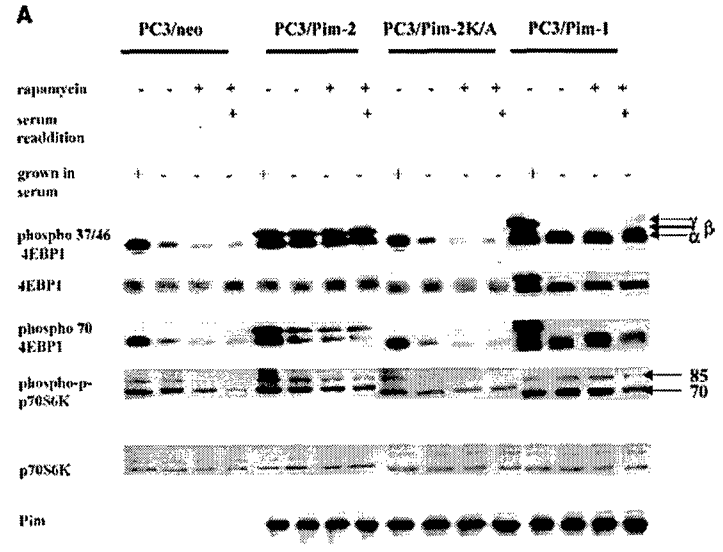


FIGURE 3A. Expression of Pim-1 or Pim-2 kinase maintains phosphorylation of key enzymes on serum starvation or treatment with rapamycin. A. PC3 cell lines containing either the neomycin-resistance gene, Pim-1, Pim-2, or Pim-2 K/A, were grown in serum, or after serum starvation for 24 hours, fresh serum-free medium was added followed by an additional 24 hours incubation with or without rapamycin (80 nmol/L). To examine the ability of serum to overcome inhibition by rapamycin (80 nmol/L), a portion of the serum-starved rapamycin (80 nmol/L)–treated cells was incubated with 20% FCS for 30 minutes before homogenization. Cellular extracts were electrophoresed on a 12% SDS-PAGE gel and then transferred to polyvinylidene difluoride membranes. Additional extracts were run to probe for S6 kinase isoforms. Arrow, phosphorylated p70 and p85 S6K protein; arrows, multiple forms of 4E-BP1doublet points at the multiple phosphorylated forms of 4E-BP1.

In addition we demonstrate that the inclusion of an RNAi to Pim-2 decreases the response to this protein and blocks the increases in 4E-BP1 phosphorylation found in this protein

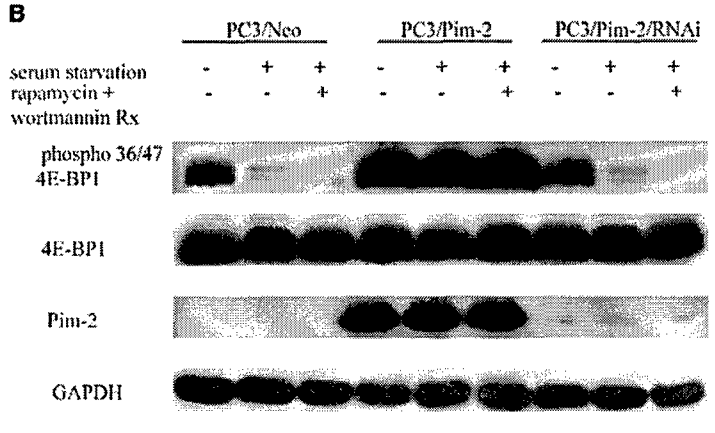


FIGURE 3B. RNA interference was used to validate the role of Pim-2 in controlling 4E-BP1 phosphorylation. PC3 cells expressing Pim-2 were transfected with an RNA interference that knocks down Pim-2 protein levels. Cells were then starved of serum for 24 hours followed by treatment with rapamycin (80 nmol/L) and wortmannin (20 nmol/L) for an additional 24 hours. Extracts were run on SDS-PAGE gels and immunoblotted with antibodies to the phosphorylated form of 4E-BP1, 4E-BP1, Pim-2, and GAPDH.FIGURE

Task 4 – The goal of this task is to examine the role of Pim in regulating tumor growth and whether Pim makes tumors more chemo resistant. We have created prostate cell lines that express Pim and tested their growth in nude mouse models. We have demonstrated that lines that express Pim grow much faster in nude mice while lines that express dominant-negative Pim are inhibited in their growth potential.

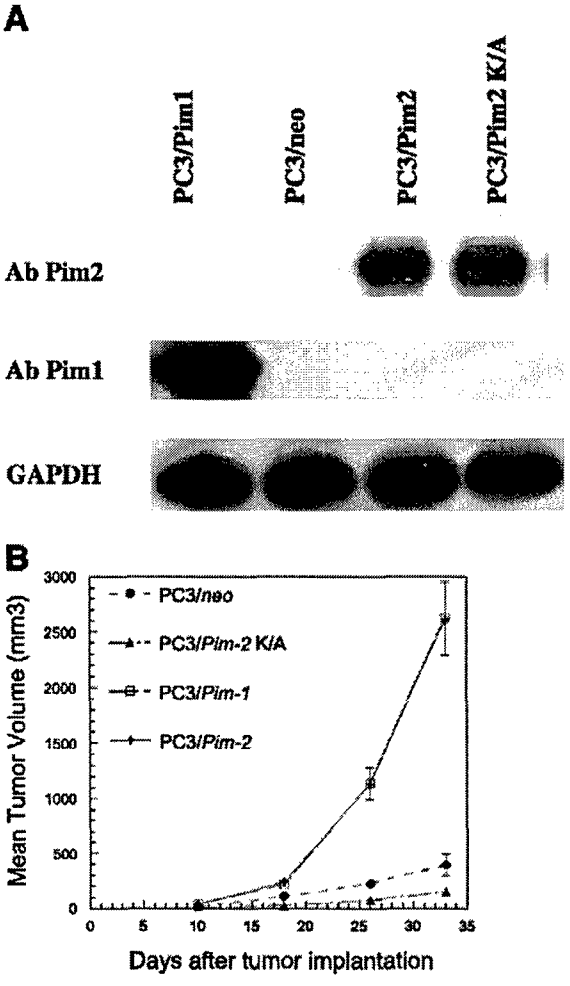
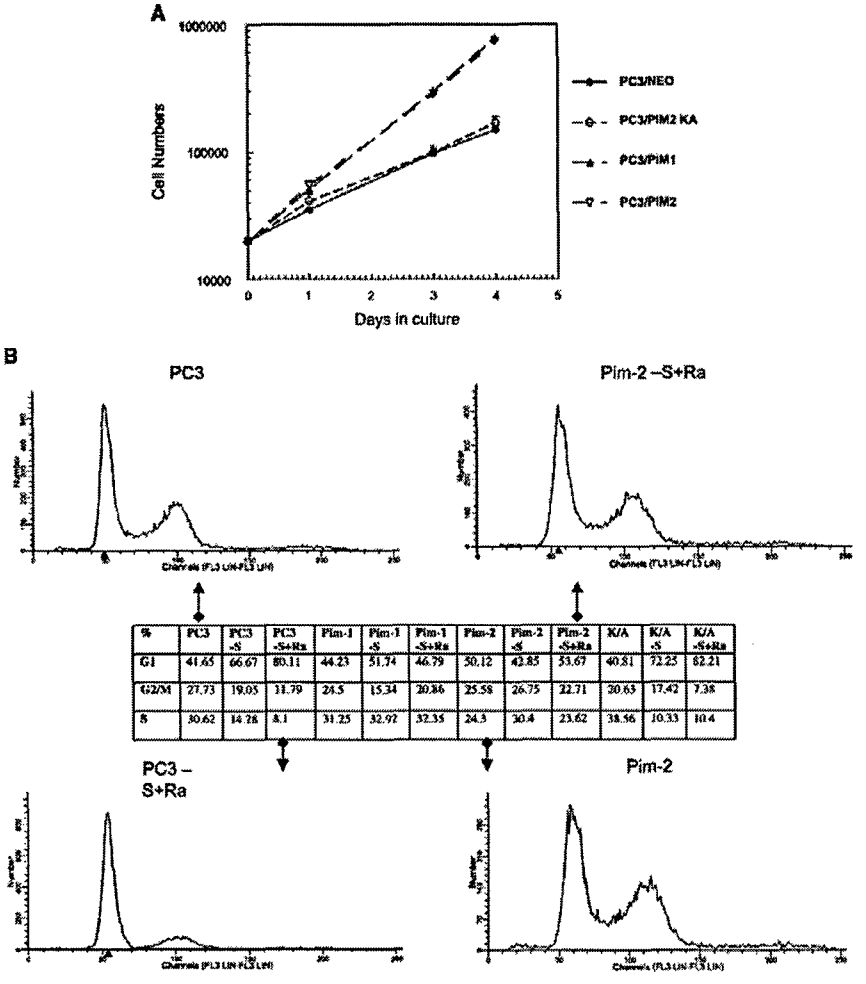


FIGURE 4. Pim kinases enhance the growth PC3 prostate tumor cells implanted in BALB/c nu/nu mice. A. Cell lines were established from PC3 cells that had been transfected with Pim-1, Pim-2, a kinase-dead mutation of Pim-2 (Pim-2 K/A), or the neomycin resistance gene. The expression of Pim-1 and Pim-2 in the cell lines is equivalent as indicated by Western blot analysis of cell lysates on 10% SDS-PAGE using monoclonal antibodies (12) to probe the blotted polyvinylidene difluoride membrane. The PC3/neo transfectant does not express levels of Pim-1 or Pim-2 that are detectable using this technique. B. PC3/neo, PC3/Pim-1, PC3/Pim-2, and PC3/Pim-2 K/A cells (2 x 106 per xenograft) were injected subcutaneously into the flanks of BALB/c nu/nu mice with five mice in each group with four tumors injected into each mouse. The tumor volume was calculated from caliper measurements in two dimensions at the indicated time points. Points, mean of 20 measurements (four tumors per mouse and five mice per group); bars, SD. The growth of Pim-1– and Pim-2–containing tumors are shown as overlapping lines.

We have further explored the ability of these cells to grow by examining them in culture. We find that Pim containing cells grow much faster than normal cells. When these cells are inhibited in their growth by rapamycin and serum starvation they continue to cycle even though they appear to be inhibited. For example, Pim 1 containing cells when treated with serum starvation and rapamycin have 32% of cells in S phase whereas control cells only have 8% of cells in the identical portion of the cell cycle.



%	PC3	PC3 -S	PC3 -S+Ra	Pim-1	Pim-1 -S	Pim-1 -S+Ra	Pim-2	Pim-2 -S	Pim-2 -S+Ra	K/A	K/A -S	K/A -S+Ra
G1	41.65	66.67	80.11	44.23	51.74	46.79	50.12	42.85	53.67	40.81	72.25	62.21
G2/M	27.73	19.05	11.79	24.5	15.34	20.86	25.58	26.75	22.71	30.63	17.42	7.38
S	30.62	14.28	8.1	31.25	32.92	32.35	24.3	30.4	23.62	38.56	10.33	10.4

FIGURE 5. Expression of Pim-1 or Pim-2 enhances the doubling time of PC3 prostate tumor cells in culture. A. The PC3 cells were plated at low density in triplicate wells and the cells harvested and counted at the indicated time points. Points, average of triplicate determinations; bars, SD. B. PC3 cell lines were starved of serum (–S) for 24 hours then either treated with rapamycin (80 nmol/L) for an additional 24 hours (+Ra) or maintained under serum-free conditions. The cells were then trypsinized, fixed in 70% ethanol, stained with propidium iodide, and subjected to DNA histogram analysis by fluorescence-activated cell sorting. The results presented are the average of triplicate determinations. However, cell cycle histograms representing individual experiments are shown.

Task 5- The next task in this proposal is to examine the effect of knocking out Pim on the level of tumorigenesis induced by c-Myc. We have encountered multiple problems in getting this aim to function. Overexpression of c-Myc is supposed to induce tumorigenesis in the mouse prostate. To examine this question we are mating mice that express c-Myc in the prostate (a gift of Charles Sawyers) with mice that are KO for Pim 1 and 2 (received from Craig Thompson). This experiment has been held up by the fact that both of these mice strains were contaminated with virus and had to be recued by foster mothering. We have now cleaned up both strains and are mating them. An additional problem is that the Pim-2 gene is on the X chromosome so only specific matings will produce heterozygotes and homozygotes. We are dealing with these complications. The third problem that we have encountered is that we have only the low myc expressive mice. The low myc mice do not get prostate cancer, but we have found that they do get PIN in dorsal and ventral glands. Thus while we will not be able to inhibit tumorigenesis, we will be able to challenge the induction of PIN in these mice.

Our second approach to this problem was to attempt to creat a transgenic mouse with Pim overexpressed in the prostate. We have created a construct with 2X probasin promoters an HA marker and the Pim gene, and gotten 12 founder Pim-1 mice and 8 founder Pim-2 mice. However, it has been difficult to identify which founder mice express Pim. We have tried both immunohistochemistry for HA and Pim. We were initially not successful. Now we are attempting RT-PCR that we will follow up with western blotting for the protein. The plan would be to mate the Pim mice with the low Myc mice and enhance the development of mouse tumors. Additional time will be necessary to make this experiment functional.

Key Research Accomplishments

- Construction of prostate cancer cell lines that express Pim proteins
- Demonstration that the expression of Pim makes tumors growth much faster while dominant negative Pim inhibits tumor take.
- The growth of Pim containing cells is faster in culture with no decrease in apoptosis.
- Pim stimulates increases in the phosphorylation of 4E-BP-1 and S6 kinase.
- RNA interference against Pim suggests that Pim controls this phosphorylation.
- Pim increases the level of c-Myc p62S phosphorylation without increasing p58 phsophorytlation.
- This phosphorylation leads to elevated levels of c-Myc even when cells are starved of serum.
- We did not see any major increase in c-Myc cDNA on QT-PCR, suggesting that the increase in levels of Myc arise from stabilization of proteins.

Reportable Outcomes

1- Chen, Wei Wei, Chan, D.C., Donald, C., Lilly, M.B., and Kraft, A.S. Pim family kinases enhance tumor growth of prostate cancer cells. Molecular Cancer Res. 3(8): 443- 451, 2005.

Conclusion

Our results clearly point to the role of Pim in regulating prostate cancer growth. Dominant negative Pim clearly inhibits the growth of these tumors. Expression of Pim makes these cells resistant to chemotherapy, especially rapamycin. We find that the mechanism of action of Pim may be related to 4E-BP1 and p70S6 phosphorylation. Because Pim increases rapamycin resistance it may be inducing phosphorylation of TOR and inhibiting rapamycin binding. We have also shown that Pim increases the levels of c-Myc. This appears to occur through the potential regulation of PP2A and the inhibition of c-Myc dephosphorylation. Since c-Myc phosphorylation controls the levels of this protein, our result that Pim inhibits the activity of PP2A is consistent with its effect on c-Myc levels. These results are exciting because they clearly suggest how Pim may play a role in transformation by stimulating protein synthesis and increasing the level of c-Myc thus stimulating prostate cancer growth.

Pim Family Kinases Enhance Tumor Growth of Prostate Cancer Cells

Wei Wei Chen,[1] Daniel C. Chan,[3] Carlton Donald,[2] Michael B. Lilly,[4] and Andrew S. Kraft[1]

[1]Hollings Cancer Center and [2]Department of Pathology, Medical University of South Carolina, Charleston, South Carolina; [3]Division of Medical Oncology, University of Colorado Health Sciences Center, Denver, Colorado; and [4]Center for Molecular Biology and Gene Therapy, Loma Linda University School of Medicine, Loma Linda, California

Abstract

Recent analyses indicate that the expression of the Pim-1 protein kinase is elevated in biopsies of prostate tumors. To identify the mechanism by which the Pim kinases may affect the growth of prostate tumors, we expressed Pim-1, Pim-2, or a kinase-dead Pim-2 protein in human PC3 prostate cancer cells. On implantation of the transfectants in nude mice, the growth of the cells expressing Pim-1 or Pim-2 was significantly faster than the growth of the control cells transfected with the neomycin-resistant gene or the kinase-dead Pim-2 protein. When grown in medium, the doubling time of the Pim-1 and Pim-2 transfectants was faster (0.75 days) than that of the control cells (1.28 days). We, therefore, examined the ability of Pim to control the phosphorylation of proteins that regulate protein synthesis. On growth factor starvation or rapamycin treatment, the Pim-1 and Pim-2 transfectants maintained their ability to phosphorylate 4E-BP1 and S6 kinase, although this phosphorylation did not occur in the control-transfected PC3 cells. We have found that the cellular levels of c-Myc were elevated in the Pim-1 and Pim-2 transfectants under these conditions. The Pim-1 and Pim-2 transfectants have lower levels of serine/threonine protein phosphatase 2A (PP2A) activity and the α- and β-subunit B56γ of the PP2A phosphatase do not coimmunoprecipitate in these cells. Thus, the effects of Pim on PP2A activity may mediate the levels of c-Myc and the phosphorylation of proteins needed for increased protein synthesis. Both of these changes could have a significant impact on tumor growth. (Mol Cancer Res 2005;3(8):443–51)

Introduction

Prostate cancer is the most common malignancy diagnosed in men in the United States. Recent evidence suggests that the Pim family of protein kinases may play a role in the development or progression of this cancer. The report of elevated levels of *pim-1* in human prostate tumors on cDNA and microarray analysis implicates the Pim family of serine/threonine kinases in the progression of human prostate tumors (1). In transgenic animal models, Pim-1 expression has been shown to be elevated in prostate tumors that are caused by overexpression of the c-*myc* oncogene (2). Although the Pim kinases have been identified as oncogenes in transgenic models, by themselves they are only weakly transforming. They have, however, been shown to greatly enhance the ability of c-*myc* gene to induce lymphomas (3, 4). The regulation of the expression of the Pim kinases and their function has been analyzed extensively in hematopoietic cells. It has been shown that the levels of Pim-1 and Pim-2 protein can be regulated by the addition of granulocyte-macrophage colony stimulating factor, interleukin-3, and interleukin-7 to normal hematopoietic cells (5) through activation of the Janus-activated kinase/signal transducers and activators of transcription pathway (6). In addition, tumor necrosis factor and Toll-like receptor ligands have been shown to induce the expression of Pim-1 (7-9). Although the overexpression of either Pim-1 or Pim-2 factor–dependent hematopoietic cells makes them resistant to apoptosis induced by interleukin-3 withdrawal (10-12), kinase-dead mutants of *pim-1* do not protect against apoptosis (13, 14). It also has been shown that the BH3 protein BAD can be phosphorylated by Pim-1 and Pim-2 and protect against apoptosis (12, 15).

Other previously described phosphorylation targets include HP-1 (16), cdc 25A phosphatase (17), and SOCS-1 (18). More recently, Pim-2 has been shown to phosphorylate the ribosomal protein 4E-BP1, causing it to dissociate from eIF-4E, which may affect protein synthesis (11). These data indicated that the ability of these protein kinases to inhibit cell death and regulate tumorigenesis is likely controlled by phosphorylation of specific target proteins. To explore the role of Pim in human prostate cancer, we have established overexpressor cell lines in PC3 cells.

Results and Discussion

To investigate the role of the Pim protein kinase family in controlling prostate cancer growth, we have created PC3 human prostate cancer cell lines that overexpress either murine Pim-1

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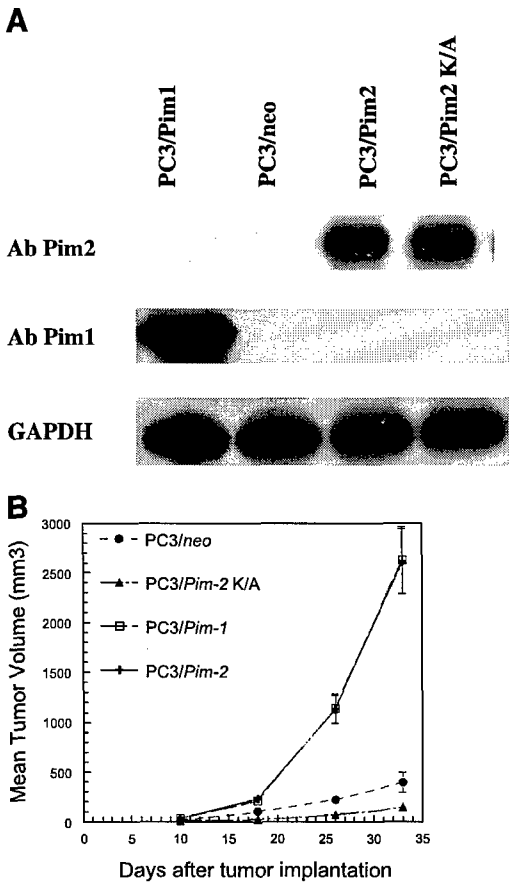


FIGURE 1. Pim kinases enhance the growth PC3 prostate tumor cells implanted in BALB/c *nu/nu* mice. **A.** Cell lines were established from PC3 cells that had been transfected with Pim-1, Pim-2, a kinase-dead mutation of Pim-2 (Pim-2 K/A), or the neomycin resistance gene. The expression of Pim-1 and Pim-2 in the cell lines is equivalent as indicated by Western blot analysis of cell lysates on 10% SDS-PAGE using monoclonal antibodies (12) to probe the blotted polyvinylidene difluoride membrane. The PC3/*neo* transfectant does not express levels of Pim-1 or Pim-2 that are detectable using this technique. **B.** PC3/*neo*, PC3/Pim-1, PC3/Pim-2, and PC3/Pim-2 K/A cells (2×10^6 per xenograft) were injected subcutaneously into the flanks of BALB/c *nu/nu* mice with five mice in each group with four tumors injected into each mouse. The tumor volume was calculated from caliper measurements in two dimensions at the indicated time points. Points, mean of 20 measurements (four tumors per mouse and five mice per group); bars, SD. The growth of Pim-1– and Pim-2–containing tumors are shown as overlapping lines.

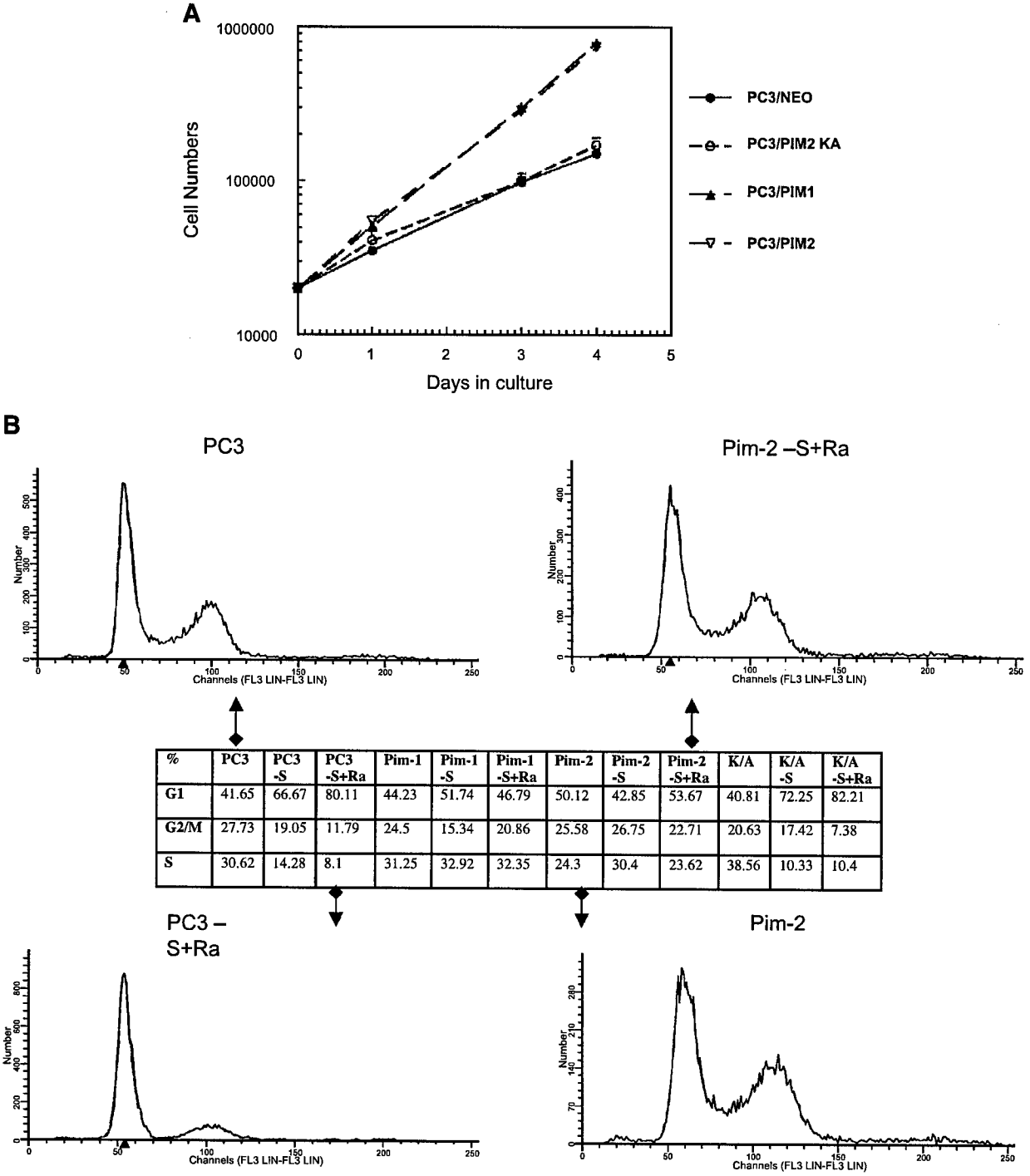
or Pim-2. At the amino acid level, human and mouse Pim-1 are 89.9% identical, whereas the Pim-2s are 86.4% identical. We have created two control cell lines by transfecting PC3 cells with either a kinase-inactive form of Pim-2 that was generated by mutating Lys^{120} to alanine using the Quik Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) or the neomycin-resistance gene (Fig. 1A). These are called PC3/Pim-1, PC3/Pim-2, PC3/Pim-2 K/A, and PC3/*neo*, respectively. To evaluate the effect of increasing Pim levels on tumor growth, the cell lines were injected subcutaneously into BALB/c *nu/nu* mice. Both PC3/Pim-1– and PC3/Pim-2–derived tumors (overlapping growth curves in Fig. 1B) grew significantly faster than PC3/*neo*–derived tumors when comparing tumor size on day 33 (paired Student's *t* test PC3/*neo* compared with PC3/Pim-1, $P < 1.36 \times 10^{-6}$; PC3/*neo* compared with PC3/Pim-2, $P < 3.33 \times 10^{-6}$). Interestingly, the kinase-dead Pim-2–transfected cells did not grow well as tumors (Fig. 1B). It is possible that kinase-dead Pim acts as a dominant-negative mutation inhibiting protein synthesis or phosphorylation of specific substrates and blocking tumor growth.

To examine whether Pim stimulates the growth rate of PC3 cells, we measured the growth rate of the Pim transfectants in tissue culture medium by plating the cells at a low density and counting cell numbers over time (Fig. 2A). Under these conditions, both the PC3/Pim-1 and PC3/Pim-2 transfectants had a doubling time of 0.75 days, whereas the doubling time of the PC3/*neo* and PC3/Pim-2 K/A cells was almost twice as long at 1.28 and 1.20 days, respectively. 4′,6-Diamidino-2-phenylindole staining of the cells disclosed that there was no significant difference in the rate of apoptosis between the different transfectants. We then examined the effect of Pim on cell growth under nutrient-limiting conditions; we starved cells of growth factors or treated them with rapamycin, which is known to block cells in the G_1 phase of the cell cycle (19). As expected, under these conditions, the PC3/*neo* and PC3/Pim-2 K/A cells were blocked in the G_1 phase of the cell cycle; however, at least over the short time period of this assay, the cell cycle distribution of the PC3/Pim transfectants was not affected significantly (Fig. 2B).

As Pim-2 has been shown to regulate the phosphorylation of proteins that control protein synthesis in hematopoietic cells (11), we investigated whether a similar mechanism might be operative in prostate tumor cells by determining the ability of overexpression of the Pim protein kinases to enhance the phosphorylation of 4E-BP1 and S6 kinase. The cell lines were either exposed to serum deprivation and nutrient starvation, treated with rapamycin, or treated with the two regimens in combination. The activity of target of rapamycin (TOR) is inhibited by the dose of rapamycin used in these experiments (20).

4E-BP1 is highly phosphorylated on Thr^{37}, Thr^{46}, Thr^{70}, Ser^{65}, Ser^{83}, Ser^{101}, and Ser^{112}. A two-stage mechanism of phosphorylation has been proposed in which Thr^{37} and Thr^{46} are phosphorylated and then allow phosphorylations to occur on Ser^{65} and Thr^{70} (21). The latter two phosphorylations are essential for inhibiting the binding of 4E-BP-1 to eIF4E (22). A number of the phosphorylations may be minor, including Ser^{83}, or constitutive, e.g., Ser^{101} and Ser^{112} (23). More highly phosphorylated 4E-BP1 has been associated with the malignant phenotype in human mammary epithelial cells (24). On SDS polyacrylamide gels, the more highly phosphorylated 4E-BP1 has been labeled γ, whereas the least phosphorylated form is denoted α (Fig. 3A; ref. 24). Multiple protein kinase pathways are thought to play a role in regulating phosphorylation of 4E-BP1. TOR activity may be required as a priming event stimulating the phosphorylation of Thr^{37} and Thr^{46}. The protein kinases that phosphorylate Ser^{65} and Thr^{70} have not been fully identified. It has been suggested that these phosphorylations may be downstream of the extracellular signal-regulated kinase or protein kinase B and directly regulated by insulin and phorbol esters (25, 26). We show that the addition of rapamycin, and to a lesser extent serum starvation, to wild-type PC3 cells markedly diminishes the phosphorylation of Thr^{37}, Thr^{46}, and Thr^{70} (Fig. 3A). In Pim-containing cells

growing in serum, the presence of either Pim-1 or Pim-2 seems to enhance the phosphorylation of 4E-BP1. In Pim-2–containing cells, neither serum starvation alone nor in combination with rapamycin decreases this phosphorylation. In Pim-1–containing PC3 cells, there is a clear decrease in phosphorylation with these treatments although there is no decrease to baseline. It is possible that Pim-1 may not function identically to Pim-2. We have found that transfected Pim-1 is



%	PC3	PC3 -S	PC3 -S+Ra	Pim-1	Pim-1 -S	Pim-1 -S+Ra	Pim-2	Pim-2 -S	Pim-2 -S+Ra	K/A	K/A -S	K/A -S+Ra
G1	41.65	66.67	80.11	44.23	51.74	46.79	50.12	42.85	53.67	40.81	72.25	82.21
G2/M	27.73	19.05	11.79	24.5	15.34	20.86	25.58	26.75	22.71	20.63	17.42	7.38
S	30.62	14.28	8.1	31.25	32.92	32.35	24.3	30.4	23.62	38.56	10.33	10.4

FIGURE 2. Expression of Pim-1 or Pim-2 enhances the doubling time of PC3 prostate tumor cells in culture. **A.** The PC3 cells were plated at low density in triplicate wells and the cells harvested and counted at the indicated time points. Points, average of triplicate determinations; bars, SD. **B.** PC3 cell lines were starved of serum (*–S*) for 24 hours then either treated with rapamycin (80 nmol/L) for an additional 24 hours (*+Ra*) or maintained under serum-free conditions. The cells were then trypsinized, fixed in 70% ethanol, stained with propidium iodide, and subjected to DNA histogram analysis by fluorescence-activated cell sorting. The results presented are the average of triplicate determinations. However, cell cycle histograms representing individual experiments are shown.

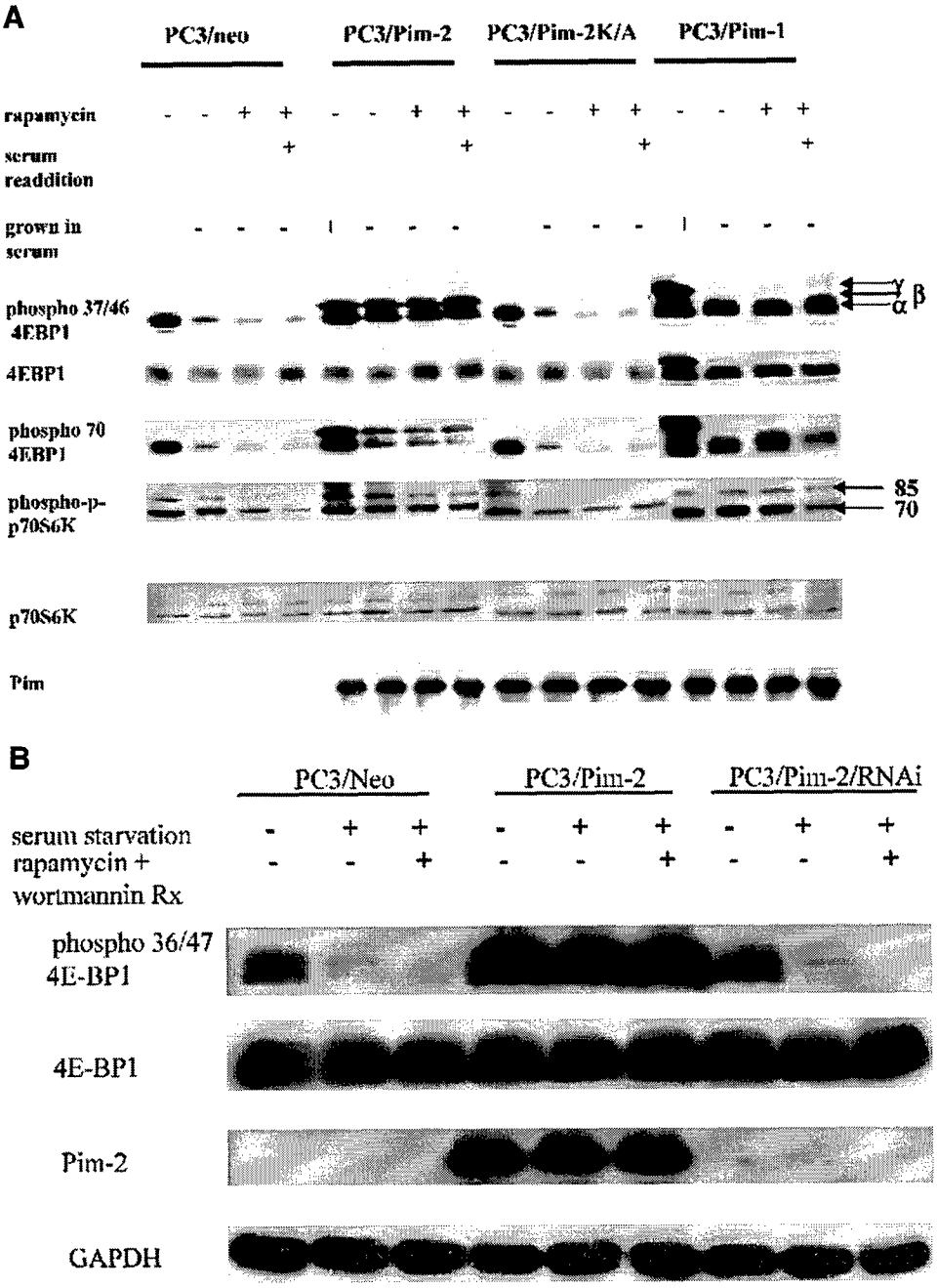


FIGURE 3. Expression of Pim-1 or Pim-2 kinase maintains phosphorylation of key enzymes on serum starvation or treatment with rapamycin. **A.** PC3 cell lines containing either the neomycin-resistance gene, Pim-1, Pim-2, or Pim-2 K/A, were grown in serum, or after serum starvation for 24 hours, fresh serum-free medium was added followed by an additional 24 hours incubation with or without rapamycin (80 nmol/L). To examine the ability of serum to overcome inhibition by rapamycin (80 nmol/L), a portion of the serum-starved rapamycin (80 nmol/L)–treated cells was incubated with 20% FCS for 30 minutes before homogenization. Cellular extracts were electrophoresed on a 12% SDS-PAGE gel and then transferred to polyvinylidene difluoride membranes. Additional extracts were run to probe for S6 kinase isoforms. Arrow, phosphorylated p70 and p85 S6K protein; arrows, multiple forms of 4E-BP1doublet points at the multiple phosphorylated forms of 4E-BP1. **B.** RNA interference was used to validate the role of Pim-2 in controlling 4E-BP1 phosphorylation. PC3 cells expressing Pim-2 were transfected with an RNA interference that knocks down Pim-2 protein levels. Cells were then starved of serum for 24 hours followed by treatment with rapamycin (80 nmol/L) and wortmannin (20 nmol/L) for an additional 24 hours. Extracts were run on SDS-PAGE gels and immunoblotted with antibodies to the phosphorylated form of 4E-BP1, 4E-BP1, Pim-2, and GAPDH.

largely located in the nucleus (27), whereas Pim-2 is thought to be cytoplasmic. Pim-1 could function in the nucleus to effect the transcription of proteins that modify the function of TOR, e.g., rictor and raptor, or specific phosphatases that regulate these proteins. Pim-2 could work directly in the cytoplasm to phosphorylate 4E-BP1 or regulate the activity of specific phosphatases.

A number of possibilities may explain these results. The Pim protein kinases may be phosphorylating Thr^{37} and Thr^{46} directly, mimicking TOR and enabling the phosphorylation of Ser^{65} and Thr^{70} by additional protein kinases. Or, Pim may be responsible for Ser^{65} and Thr^{70} phosphorylation. Other possibilities to explain Pim-2 protein kinase activity include regulation of the rapamycin-insensitive TOR complex bound to the protein rictor (28) or modulation of the protein phosphatase 2A (PP2A) activity that is an important regulatory protein in the TOR pathway in yeast (29). The S6 kinase message encodes two isoforms, p70 and p85 (30). Like 4E-BP1, S6 kinase is activated by a two-step mechanism by which TOR phosphorylates multiple sites (Ser^{411}, Ser^{418}, Ser^{424}, and Thr^{421}), which is then followed by an activating phosphorylation (Thr^{229}) by PKD1 protein kinase (31). We find that amino acid starvation

and rapamycin treatment markedly decreases the phosphorylation of both p70 and p85 S6 kinases (Fig. 3A). In contrast, Pim protein kinases maintain these two kinases in the phosphorylated and active form. The activity of the Pim kinases may be explained by the ability of these enzymes to phosphorylate and activate a subset of TOR protein complexes, phosphorylate S6 kinase directly, and regulate the dephosphorylation of this protein kinase or the activity and specificity of PKD1.

Using RNA interference directed at Pim-2 that has been validated in other cell lines, it is shown that the knockdown of Pim-2 protein levels in PC3 Pim-2 cells markedly inhibits the phosphorylation of 4E-BP1 (Fig. 3B; ref. 11). However, it had no effect on the total cellular levels of 4E-BP1. This knockdown occurs in the presence of rapamycin and wortmannin, where Pim seems to be regulating 4E-BP1 phosphorylation. In contrast, this small interfering RNA treatment had no effect on the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in these cells. These data confirm that Pim-2 can modulate the phosphorylation of proteins that play a crucial role in controlling protein synthesis in prostate cancer. We also show the ability of overexpressed Pim-1 compared with Pim-2 to modulate the phosphorylation in a similar if not identical fashion. This regulation of protein synthesis may contribute significantly to the ability of Pim protein kinases to enhance tumor growth.

Cap-dependent translation controls the level of multiple proteins, including c-Myc, a protein that seems to collaborate with Pim (32, 33). Under the conditions described above, we evaluated the cellular levels of c-Myc and found that the level of this protein is higher in the Pim-1 and Pim-2 transfectants than in control PC3/*neo* cells (Fig. 4A). Growth factor

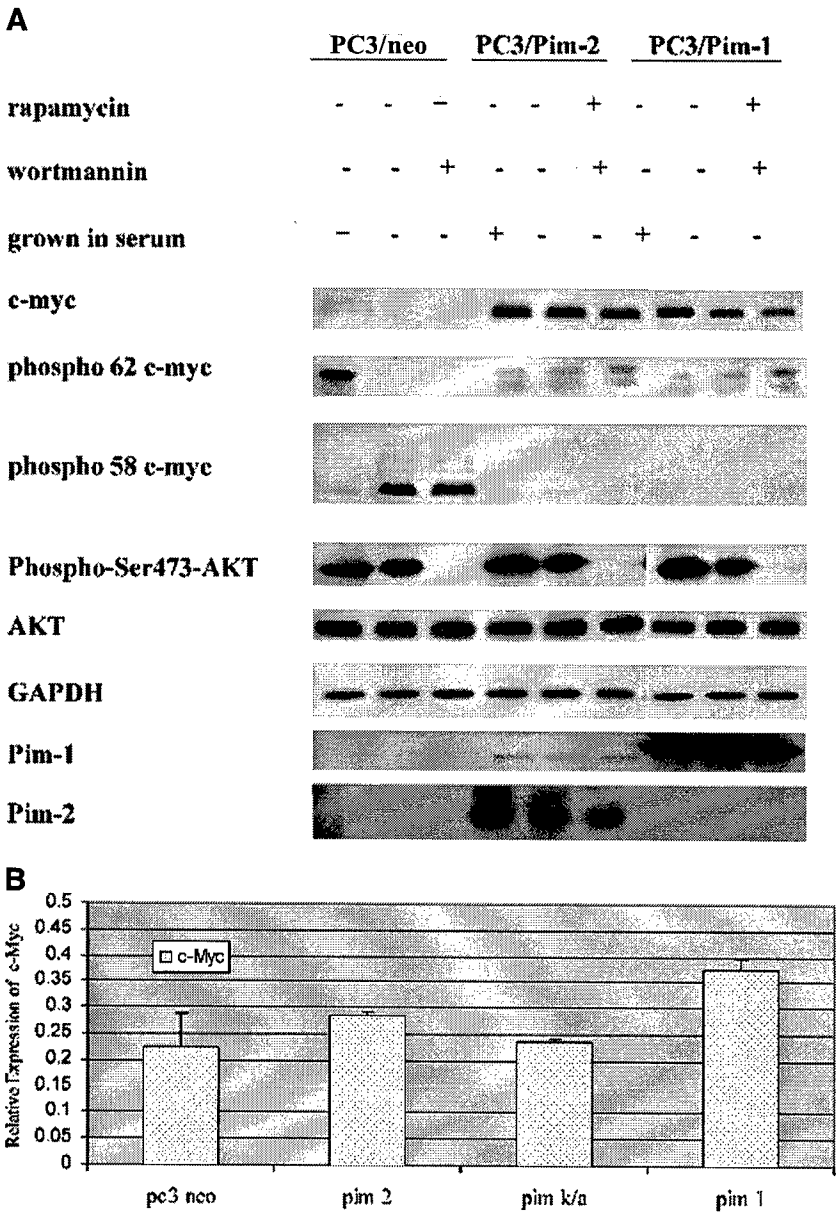


FIGURE 4. Regulation of c-Myc levels in Pim-containing PC3 cells. **A.** To evaluate c-Myc levels, PC3/*neo*, PC3/Pim-1, and PC3/Pim-2 were grown in RPMI, including 10% fetal bovine serum, then serum-starved for 24 hours and treated with rapamycin (80 nmol/L) and wortmannin (20 nmol/L) for an additional 24 hours before harvesting. Extracts were run on SDS-PAGE gels. The membrane was stripped and probed with antibodies specific for c-Myc, phospho-Ser^{62}, phospho-Thr^{58}, phospho-Ser^{473} AKT, AKT, and GAPDH, a loading control. **B.** To measure the level of *c-Myc* mRNA in PC3 cells, mRNA was extracted as described in Materials and Methods and subjected to quantitative reverse transcription-PCR. The level of c-*myc* mRNA was compared with GAPDH and expressed as a ratio for each sample. The experiment was repeated with three individual RNA samples and was done in triplicate. Columns, mean; bars, SD.

starvation or rapamycin and wortmannin treatment decreased the level of c-Myc in control cells. Likewise, these treatments inactivated the endogenous AKT phosphorylation that is elevated in PC3 cells. These manipulations had little effect on the level of c-Myc in the Pim-containing cells. Phosphorylation of c-Myc on Ser^{62} by the action of the extracellular signal-regulated kinase pathway stabilizes the protein, whereas GSK-3β phosphorylation of Thr^{58} targets the protein to ubiquitin-mediated degradation (34, 35). Serum starvation of PC3/*neo* cells or treatment with wortmannin and rapamycin increased phosphorylation of Thr^{58} while decreasing phosphorylation of Ser^{62}. In the Pim-1 and Pim-2 transfectants, these treatments had no significant effect and Ser^{62} remained phosphorylated. The combined treatment with rapamycin and wortmannin clearly inhibited the activity of the AKT protein kinase in all cell lines (Fig. 4A). It has been shown previously that c-Myc in which Ser^{62} is phosphorylated, but not unphosphorylated c-Myc, functions to transform normal human fibroblasts transfected with human telomerase and Ras^{G12V} and is responsible for activation of the E2F promoter (36). Activated c-Myc can increase the transcription of eIF-4E, further enhancing protein synthesis (37-39). Thus, it is possible that modulation of the phosphorylation and, hence, the levels of c-Myc by Pim may be important in its mechanism of action and the regulation of tumor cell growth.

To evaluate the possibility that the mechanism by which Pim regulates c-Myc protein levels is through increasing c-Myc mRNA, we did quantitative PCR. The levels of c-Myc were normalized to GAPDH and the experiment was repeated on three independent samples. The results show that control cells, Pim-2, and Pim-2 K/A cells have very similar levels of c-Myc mRNA (Fig. 4B). In contrast, we find that the Pim-1 cells seem to have increased levels of c-Myc mRNA when compared with the other three cell lines. Student's *t* test comparing the levels of c-Myc with the control cells shows a *P* value of 0.03, suggesting only modest statistical difference. It has been suggested that the biological activities of Pim-1 and Pim-2 are different (11). Pim-2 functions in the cytoplasm and stimulates the Cot kinase to enhance the activity of nuclear factor-κB (40). In contrast, we have found that Pim-1, when transfected into 293T cells or in stained human Burkitt's lymphoma cells, is located in the nucleus (27). Thus, it is possible that Pim-1 could regulate c-Myc mRNA levels in a different manner than Pim-2.

The phosphorylation of serine PP2A results in the dephosphorylation of c-Myc on Ser^{62}, thereby allowing its Thr^{58}-directed protein ubiquination and degradation (36). The degradation of c-Myc blocks its cell cycle– and growth-promoting activity, inhibiting its ability to regulate transcription. The serine phosphatases also have been shown to play an important role in regulating protein synthesis in yeast (41). Pim has been shown to coimmunoprecipitate with the catalytic subunit of PP2A (42), suggesting that Pim might regulate the activity of this phosphatase. Using a colorimetric assay for PP2A that was validated by demonstrating its inhibition with okadaic acid and sodium fluoride (Fig. 5A), we measured PP2A activity in the four PC3 cell lines (Fig. 5A). This experiment showed that the Pim-1 and Pim-2 transfectants exhibited a significantly lower level of PP2A activity than the PC3/*neo*

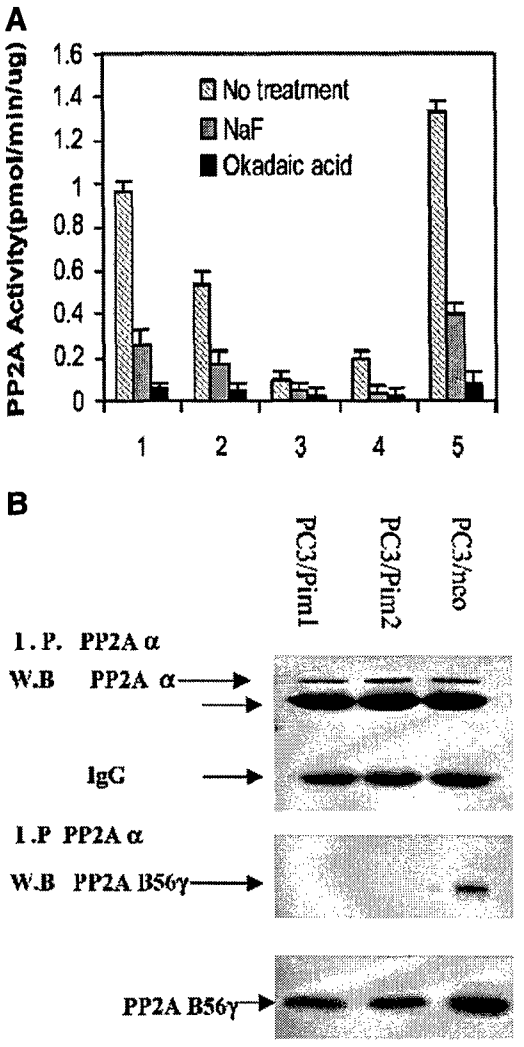


FIGURE 5. Expression of Pim-1 or Pim-2 regulates PP2A activity in PC3 prostate tumor cells. **A.** Cells (2.5×10^6) were harvested in Tris-HCl–buffered saline, centrifuged, and then sonicated in 500 μL buffer [10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, and 0.02% sodium azide]. The phosphatase assay was carried out as described in Materials and Methods. As controls, either sodium fluoride (50 mmol/L) or okadaic acid (5 μmol/L) was added to cell extracts before beginning the assay. Columns, mean of triplicate determinations; bars, SD. 1, PC3/neo; 2, PC3/Pim-2 K/A; 3, PC3/Pim-2; 4, PC3/Pim-1; 5, PP2A catalytic subunit. **B.** 1×10^6 cells were lysed in 50 mmol/L Tris-HCl (pH 7.4); 150 mmol/L NaCl; 1 mmol/L EDTA; 1 mmol/L phenylmethylsulfonyl fluoride; and 1 μg/mL each of aprotinin, leupeptin, and pepstatin. The extracts were immunoprecipitated with an antibody to the α-subunit of PP2A. The immunoprecipitates were run on a 10% SDS-PAGE gel, transferred to polyvinylidene difluoride membranes, and probed with antibodies to the α-subunit of PP2A or an antibody to the β-subunit B56γ. Arrows, α- and β-subunits and the IgG light and heavy chains.

control cells. Interestingly, the PC3/Pim-2 K/A cells had a slightly lower level of phosphatase activity than PC3/*neo* controls, possibly suggesting that this protein could interact with PP2A in the absence of kinase activity. Recent evidence (43) has shown that polyoma small-T antigen inhibits PP2A activity by causing the dissociation of the catalytic and regulatory subunits of this phosphatase. To investigate this possibility, we immunoprecipitated the PP2A α-subunit and

then Western blotted with an antibody to the β-component of the complex, B56γ. Our results show that the overexpression of Pim-1 or Pim-2 compared with the PC3/*neo* cells caused apparent disassociation of these two subunits. However, the total levels of B56γ do not change in these cells (Fig. 5B). This dissociation could function to inhibit PP2A activity, and thus indirectly regulate the phosphorylation of c-Myc as seen in Fig. 4A. By modulating c-Myc phosphorylation and thus its degradation, Pim protein kinases can function to enhance the activity of c-Myc and potentially to stimulate transit through the cell cycle.

The TOR pathway and phosphorylation of 4E-BP1 are critical to the growth of tumors (26, 44). Multiple enzyme abnormalities in tumor cells affect this pathway, including increased activity of AKT (45, 46) and mutation of TSC1 and TSC2 (47, 48). Normally, the phosphorylation of the 4E-BP1 protein and the p70S6 kinase is stimulated by the AKT protein kinase through a TOR-dependent pathway (19, 49). The phosphorylation of p70S6 kinase causes phosphorylation of the S6 protein and stimulates protein synthesis. Phosphorylation of 4E-BP1 on Thr^{37} and Thr^{46} potentiates the further phosphorylation of 4E-BP1 on Ser^{70}, dissociating this protein from eIF-4E. Free eIF-4E stimulates Cap-dependent protein translation of a large number of proteins, including c-Myc. Pim family members can phosphorylate the sequence RRRLS/T (50), which is similar to that modified by AKT, RXRXXS/T (51), suggesting that they could affect similar pathways. However, the observation that rapamycin blocked AKT-mediated TOR phosphorylation, but not Pim-mediated phosphorylation of 4E-BP1, suggests that the mechanisms by which Pim affect the pathway differ from AKT. It may be inferred, however, that the effect of Pim on PP2A could also be crucial in regulating protein synthesis.

There has been recent interest in the development of rapamycin analogues as treatment regimens for tumors in which PTEN is deleted (52-56) or as drugs that could sensitize tumors to other chemotherapeutic agents (20). Clearly, tumors that overexpress Pim would be resistant to both of these approaches. Knowledge of the level of overexpression of Pim in tumors may help in planning chemotherapy regimens. Additionally, targeting the Pim kinases with small molecule inhibitors may play a critical role in cancer therapy.

Materials and Methods

Materials

Rapamycin, wortmannin, and antibodies to wild-type and phosphorylated forms of 4E-BP1, phospho-Ser^{473} AKT, AKT, and p70S6K were purchased from Cell Signaling Technologies (Beverly, MA). Okadaic acid was obtained from Invitrogen (Carlsbad, CA). Monoclonal Pim-2 and Pim-1 antibody were generated by immunization with a 20–amino acid peptide corresponding to the carboxyl terminus of the Pim-2 and Pim-1 protein. Antibodies were purified by affinity chromatography using peptides covalently liked to 4B Sepharose (Amersham, Piscataway, NJ). Anti-flag M2 was purchased from Eastman Kodak Company (New Haven, CT). Additional antibodies used include c-Myc (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Ser^{62} (Abcam, Cambridgeshire, United Kingdom), phospho-Thr^{58} (Cell Signaling), α-subunit of PP2A (6F9; Covance, Berkeley, CA), an antibody to the β-subunit B56γ a gift of Dr. William Hahn (Harvard Medical School, Boston, MA), and GAPDH (Chemicon, Temecula, CA), a loading control.

Plasmids

The murine *pim-1* and *pim-2* cDNAs were PCR amplified using primers with *Eco*RI and *Xho*I restriction site at their ends and cloned into the PCDNA.3.1 expression vector (Invitrogen). A kinase-dead mutant form of pim-2 expression vector (*pimK/A*) was generated by mutating Lys^{120} to alanine using the Quik Change Site-Directed Mutagenesis kit (Stratagene).

Cell Culture and Transfection

PC3 cell lines were maintained in RPMI 1640 containing 10% FCS penicillin/streptomycin. PC3 cells were transfected using Effectene (Qiagen, Valencia, CA) according to the instructions of the manufacturer. For derivation of cell lines after 48 hours, the culture medium was switched to the same medium supplemented with 1,000 μg/mL Geneticin. For rapamycin treatment of stable and transiently transfected cells, all cell lines were first placed in serum-free medium for 24 hours and then fresh serum-free medium was added before addition of 80 nmol/L rapamycin.

Western Blot Analysis

Cell extracts were prepared by lysing cells in a buffer containing 20 mmol/L Tris (pH 7.5),150 mmol/L NaCl, 1 mmol/L EDTA,1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium PPi,1 mmol/L β-glycerolphosphate, 0.5% NP40, 1 mmol/L Na_3VO_4, 1 μg/mL leupeptin, and protease inhibitor cocktail (Sigma Chemical, St. Louis, MO). The lysates were incubated on ice for 15 minutes followed by centrifugation for 10 minutes at 4°C. To the supernatant was added with 5× Ficoll and the mixture was heated at 100°C for 5 minutes. The cell extracts were resolved by 12% SDS-PAGE and then transferred to Immobilon-P membrane filters (Millipore, Bedford, MA).

Cell Cycle Analysis

Twenty-four hours after culturing prostate cancer cell lines in normal medium, they were transferred to serum-free medium and cultured for 24 additional hours. The tumor cells were then treated with 80 nmol/L rapamycin or DMSO for 18 hours and subjected to propidium iodide staining. The percentage of cells in different phases of the cycle was determined by fluorescence-activated cell sorting analysis.

Tumor Growth Curve

Subcutaneous tumor xenografts were established in nude mice by injecting tumor cells ($2 \times 10^{+6}$) subcutaneously in four locations on each mouse. Seven days after transplantation, tumor measurements were begun in a blinded fashion with five mice per group or 20 total tumors.

Phosphatase Assay

Cells (2.5×10^6) were harvested in Tris-HCl–buffered saline, centrifuged, and then sonicated in 500 μL buffer

[10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, and 0.02% sodium azide]. A 250 μL aliquot of the homogenate was applied to a G25 column to decrease the levels of free phosphate, and the flow through subjected to a PP2A colorimetric assay following the instructions of the manufacturer (Promega, Madison WI) and using the PP2A-specific buffer. As controls, either sodium fluoride (50 mmol/L) or okadaic acid (5 μmol/L) was added to cell extracts before beginning the assay.

RNA Interference

A short interfering RNA for Pim-2 (5′-GGGATAGATGGACATCTGTTGAA-3′) that had been previously validated (11) was purchased from Ambion (Austin, TX) and transfected into PC3 cells at a concentration of 100 pmol using LipofectAMINE 2000 (Invitrogen). Thirty-six hours later, the serum was withdrawn for 24 hours and then rapamycin (80 nmol/L) and wortmannin (20 nmol/L) were added for an additional 48 hours.

RNA Isolation and Quantitative Reverse Transcription-PCR

Total RNA (0.5 μg per reaction) was reverse transcribed into cDNA utilizing random primers (Promega). AMV Reverse Transcriptase II enzyme (500 units per reaction; Promega) was used for first-strand synthesis and Tfl DNA Polymerase for second-strand synthesis (500 units per reaction; Promega) as per the protocol of the manufacturer. In each case, 50 pg of cDNA was used per ensuing PCR reaction. Two-step quantitative reverse transcription-PCR was done on cDNA generated using the MultiScribe Reverse Transcriptase from the TaqMan Reverse Transcription System and the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA).

The primer pair for c-Myc was generated from the published c-Myc sequence (accession no. NM_002467). Forty cycles of PCR were done under standard conditions using an annealing temperature of 56°C. In addition, *GAPDH* was amplified as a housekeeping gene to normalize the initial content of total cDNA. Here, c-Myc expression was calculated as the relative expression ratio between c-Myc and GAPDH and was compared for each condition. As a negative control, quantitative reverse transcription-PCR reactions without cDNA template were also done. All reactions were run thrice in triplicate.

Acknowledgments

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